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(54) Title: CHIMERIC FLAVIVIRUS VACCINES

(57) Abstract

A chimeric live, infectious, attenuated virus, containing a yellow fever virus, in which the nucleotide sequence for a prM–E protein is either deleted, truncated, or mutated, so that functional prM–E protein is not expressed, and integrated into the genome of the yellow fever virus, a nucleotide sequence encoding a prM–E protein of a second, different flavivirus, so that the prM–E protein of the second flavivirus is expressed.

CHIMERIC FLAVIVIRUS VACCINES

Background of the Invention

This invention relates to infectious, attenuated viruses useful as vaccines against diseases caused by flaviviruses.

Several members of the flavivirus family pose current or potential threats to global public health. For example, Japanese encephalitis is a significant public health problem involving millions of at risk individuals in the Far East. Dengue virus, with an estimated annual incidence of 100 million cases of primary dengue fever and over 450,000 cases of dengue hemorrhagic fever worldwide, has emerged as the single most important arthropod-transmitted human disease. Other flaviviruses continue to cause endemic diseases of variable nature and have the potential to emerge into new areas as a result of changes in climate, vector populations, and environmental disturbances caused by human activity. These flaviviruses include, for example, St. Louis encephalitis virus, which causes sporadic, but serious acute disease in the midwest, southeast, and western United States; West Nile virus, which causes febrile illness, occasionally complicated by acute encephalitis, and is widely distributed throughout Africa, the Middle East, the former Soviet Union, and parts of Europe; Murray Valley encephalitis virus, which causes endemic nervous system disease in Australia; and Tick-borne encephalitis virus, which is distributed throughout the former Soviet Union and eastern Europe, where its ixodid tick vector is prevalent and responsible for a serious form of encephalitis in those regions.

Hepatitis C virus (HCV) is another member of the flavivirus family, with a genome organization and replication strategy that are similar, but not

virions by a furin-like cellular protease (Stadler *et al.*, J. Virol. 71:8475-8481, 1997), which is necessary to activate hemagglutinating activity, fusogenic activity, and infectivity of virions. The M protein is cleaved from its precursor protein (prM) after the consensus sequence R-X-R/K-R (X is variable), and incorporated into the virus lipid envelope together with the E protein.

Cleavage sequences have been conserved not only within flaviviruses, but also within proteins of other, unrelated viruses, such as PE2 of murine coronaviruses, PE2 of alphaviruses, HA of influenza viruses, and p160 of retroviruses. Cleavage of the precursor protein is essential for virus infectivity, but not particle formation. It was shown that, in case of a TBE-dengue 4 chimera, a change in the prM cleavage site resulted in decreased neurovirulence of this chimera (Pletnev *et al.*, J. Virol. 67:4956-4963, 1993), consistent with the previous observation that efficient processing of the prM is necessary for full infectivity (Guirakhoo *et al.*, 1991, *supra*, 1992, *supra*; Heinz *et al.*, Virology 198:109-117, 1994). Antibodies to prM protein can mediate protective immunity, apparently due to neutralization of released virions that contain some uncleaved prM. The proteolytic cleavage site of the PE2 of VEE (4 amino acids) was deleted by site-directed mutagenesis of the infectious clone (Smith *et al.*, ASTMH meeting, December 7-11, 1997). Deletion mutants replicated with high efficiency and PE2 proteins were incorporated into particles. This mutant was evaluated in non-human primates and shown to cause 100% seroconversion and protected all immunized monkeys from a lethal challenge.

Summary of the Invention

The invention features chimeric, live, infectious, attenuated viruses that are each composed of:

Japanese Encephalitis (JE), Dengue (DEN, *e.g.*, any of Dengue types 1-4), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE), and Hepatitis C (HCV) viruses. Additional flaviviruses for use as the second flavivirus include Kunjin virus, Central European Encephalitis virus, Russian Spring-Summer Encephalitis virus, Powassan virus, Kyasanur Forest Disease virus, and Omsk Hemorrhagic Fever virus. In a preferred chimeric virus of the invention, the prM-E protein coding sequence of the second flavivirus is substituted into the prM-E protein coding sequence of the live yellow fever virus. In a preferred chimeric virus, the prM-E protein coding sequence is derived from an attenuated virus strain, such as a vaccine strain. Also, as is described further below, the prM portion of the protein can contain a mutation that prevents cleavage to generate mature membrane protein.

Also included in the invention are methods of preventing or treating flavivirus infection in a mammal, such as a human, by administering a chimeric flavivirus of the invention to the mammal; use of the chimeric flaviviruses of the invention in the preparation of medicaments for preventing or treating flavivirus infection; nucleic acid molecules encoding the chimeric flaviviruses of the invention; and methods of manufacturing the chimeric flaviviruses of the invention.

The invention provides several advantages. For example, because they are live and replicating, the chimeric viruses of the invention can be used to produce long-lasting protective immunity. Because the viruses have the replication genes of an attenuated virus (*e.g.*, Yellow Fever 17D), the resulting chimeric virus is attenuated to a degree that renders it safe for use in humans.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

Detailed Description

The invention provides chimeric flaviviruses that can be used in vaccination methods against flavivirus infection. Construction and analysis of chimeric flaviviruses of the invention, such as chimeras of yellow fever virus and Japanese Encephalitis (JE), Dengue types 1-4 (DEN 1-4), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE), and Hepatitis C (HCV) viruses are described as follows.

Flavivirus proteins are produced by translation of a single, long open reading frame (encoding, *i.a.*, the structural proteins, capsid (C), pre-membrane (pr-M), and envelope (E), as well as non-structural proteins) and a complex series of post-translational proteolytic cleavages. The chimeric flaviviruses of the invention, as is discussed above, include those in which the pr-M and E proteins of one flavivirus have been replaced by the pr-M and E proteins of another flavivirus. Thus, creation of these chimeric flaviviruses involves the generation of novel junctions between the capsid and pre-membrane proteins, and the envelope protein and the non-structural region (NS1), of two different flaviviruses. Cleavage between each of these sets of proteins (C and pr-M, and E and NS1) occurs during the natural proteolytic processing of flavivirus proteins, and requires the presence of signal sequences flanking the junctions of the cleavage sites.

In the chimeric flaviviruses of the invention, it is preferred that the signal sequences of the viruses making up the chimeras are substantially maintained, so that proper cleavage between the C and pr-M and E and NS1 proteins can efficiently take place. These signal sequences have been maintained in the chimeras described below. Alternatively, any of numerous known signal sequences can be engineered to link the C and pre-M or E and

JE sequences from the start of the prM protein (nucleotide 478, amino acid 128) through the E/NS1 cleavage site (nucleotide 2,452, amino acid 817). In addition to cloning of JE cDNA, several steps were required to introduce or eliminate restriction sites in both the YF and JE sequences to permit *in vitro* ligation. The structure of the template for regenerating chimeric YF (C)/JE (prM-E) virus is shown in Fig. 4. A second chimera, encoding the entire JE structural region (C-prM-E) was engineered using a similar strategy.

Molecular Cloning of the JE Virus Structural Region

Clones of authentic JE structural protein genes were generated from the JE SA_{14}-14-2 strain (JE live, attenuated vaccine strain), because the biological properties and molecular characterization of this strain are well-documented (see, *e.g.*, Eckels *et al.*, Vaccine 6:513-518, 1988; JE SA_{14}-14-2 virus is available from the Centers for Disease Control, Fort Collins, Colorado and the Yale Arbovirus Research Unit, Yale University, New Haven, Connecticut, which are World Health Organization-designated Reference Centers for Arboviruses in the United States). JE SA_{14}-14-2 virus at passage level PDK-5 was obtained and passaged in LLC-MK_2 cells to obtain sufficient amounts of virus for cDNA cloning. The strategy we used involved cloning the structural region in two pieces that overlap at an *Nhe*I site (JE nucleotide 1,125), which can then be used for *in vitro* ligation.

RNA was extracted from monolayers of infected LLC-MK_2 cells and first strand synthesis of negative sense cDNA was carried out using reverse transcriptase with a negative sense primer (JE nucleotide sequence 2,456-71) containing nested *Xba*I and *Nar*I restriction sites for cloning initially into pBluescript II KS(+), and subsequently into YFM5.2(*Nar*I), respectively. First strand cDNA synthesis was followed by PCR amplification of the JE sequence

positive sense primer corresponding to YF5'3'IV nucleotides 6,625-6,639 to generate PCR fragments that were then used as negative sense PCR primers in conjunction with a positive sense primer complementary to the pBluescript vector sequence upstream of the *EcoR*I site, to amplify the JE sequence (encoded in reverse orientation in the pBluescript vector) from nucleotide 477 (N-terminus of the prM protein) through the *Nhe*I site at nucleotide 1,125. The resulting PCR fragments were inserted into the YF5'3'IV plasmid using the *Not*I and *EcoR*I restriction sites. This construct contains the SP6 promoter preceding the YF 5'-untranslated region, followed by the sequence: YF (C) JE (prM-E), and contains the *Nhe*I site (JE nucleotide 1,125) required for *in vitro* ligation.

Engineering YFM5.2 and YF5'3'IV to Contain Restriction Sites for in vitro *Ligation*

In order to use the *Nhe*I site within the JE envelope sequence as a 5' *in vitro* ligation site, a redundant *Nhe*I site in the YFM5.2 plasmid (nucleotide 5,459) was eliminated. This was accomplished by silent mutation of the YF sequence at nucleotide 5,461 (T→C; alanine, amino acid 1820). This site was incorporated into YFM5.2 by ligation of appropriate restriction fragments and introduced into YFM5.2(*Nar*I)/JE by exchange of an *Nsi*I/*Nar*I fragment encoding the chimeric YF/JE sequence.

To create a unique 3' restriction site for *in vitro* ligation, a *BspE*I site was engineered downstream of the *Aat*II site normally used to generate full-length templates from YF5'3'IV and YFM5.2. (Multiple *Aat*II sites are present in the JE structural sequence, precluding use of this site for *in vitro* ligation.) The *BspE*I site was created by silent mutation of YF nucleotide 8,581 (A→C; serine, amino acid 2,860) and was introduced into YFM5.2 by exchange of

YFM5.2/JE are digested with *NheI*/*BspE*I and *in vitro* ligation is performed using 50 nanograms of purified fragments in the presence of T4 DNA ligase. The ligation products are linearized with *Xho*I to allow run-off transcription. SP6 transcripts are synthesized using 50 nanograms of purified template, quantitated by incorporation of ^{3}H-UTP, and integrity of the RNA is verified by non-denaturing agarose gel electrophoresis. Yields range from 5 to 10 micrograms of RNA per reaction using this procedure, most of which is present as full-length transcripts. Transfection of RNA transcripts in the presence of cationic liposomes is carried out as described by Rice *et al.* (*supra*) for YF17D. In initial experiments, LLC-MK_2 cells were used for transfection and quantitation of virus, since we have determined the permissiveness for replication and plaque formation of the parental strains of YF and JE. Table 1 illustrates typical results of transfection experiments using Lipofectin (GIBCO/BRL) as a transfection vehicle. Vero cell lines have also been used routinely for preparation of infectious virus stocks, characterization of labeled proteins, and neutralization tests.

Nucleotide Sequencing of Chimeric cDNA Templates

Plasmids containing the chimeric YF/JE cDNA were subjected to sequence analysis of the JE portion of the clones to identify the correct sequences of the SA_{14}-14-2 and Nakayama envelope protein. The nucleotide sequence differences between these constructs in comparison to the reported sequences (McAda *et al.*, *supra*) are shown in Table 2.

Structural and Biological Characterization of Chimeric YF/JE Viruses

The genomic structure of chimeric YF/JE viruses recovered from transfection experiments was verified by RT/PCR-based analysis of viral RNA

monoclonal antibodies in this analysis. Plaque reduction neutralization testing (PRNT) was performed on the chimeric viruses and the YF and JE SA_{14}-14-2 viruses using YF and JE-specific hyperimmune ascitic fluid (ATCC) and YF-specific purified IgG (monoclonal antibody 2E10). Significant differences in the 50% plaque reduction titer of these antisera were observed for the chimeras when compared to the control viruses in these experiments (Table 3). Thus, epitopes required for neutralization are expressed in the infectious chimeric YF/JE viruses.

Growth Properties in Cell Culture

The growth capacity of the chimeras has been examined quantitatively in cell lines of both primate and mosquito origin. Fig. 2 illustrates the cumulative growth curves of the chimeras on LLC-MK_2 cells after low multiplicity infection (0.5 plaque-forming units/cell). In this experiment, YF5.2iv (cloned derivative) and JE SA_{14}-14-2 (uncloned) viruses were used for comparison. Both chimeric viruses reached a maximal virus yield of approximately one log higher than either parental virus. In the case of the YF/JE SA_{14}-14-2 chimera, the peak of virus production occurred 12 hours later than the YF/JE Nakayama chimera (50 hours vs. 38 hours). The YF/JE Nakayama chimera exhibited considerably more cytopathic effects than the YF/JE SA_{14}-14-2 chimera on this cell line. A similar experiment was carried out in C6/36 cells after low multiplicity infection (0.5 plaque-forming units/cell). Fig. 2 also illustrates the growth kinetics of the viruses in this invertebrate cell line. Similar virus yields were obtained at all points used for virus harvest in this experiment, further substantiating the notion that chimeric viruses are not impaired in replication efficiency.

week post-inoculation. No mortality or illness was observed among mice receiving either the JE SA_{14}-14-2 parent or the chimera. The inocula used for the experiments were titered at the time of injection and a subgroup of the surviving mice were tested for the presence of neutralizing antibodies to confirm that infection had taken place. Among those tested, titers against the JE SA_{14}-14-2 virus were similar for animals receiving either this strain or the chimera.

The results of additional experiments investigating the neurovirulence of the YF/JE SA_{14}-14-2 chimera in mice are illustrated in Table 4. In these experiments, all of the mice inoculated with YF5.2iv died within 7-8 days. In contrast, none of the mice inoculated with YF/JE SA_{14}-14-2 died during two weeks of post-inoculation observation.

The results of experiments investigating the neuroinvasiveness and pathogenesis of YF/JE chimeras are illustrated in Table 5. In these experiments, the chimeric viruses were inoculated into 3 week old mice at doses varying between 10,000 and 1 million plaque-forming units via the intraperitoneal route. None of the mice inoculated with YF/JE Nakayama or YF/JE SA_{14}-14-2 died during three weeks of post-inoculation observation, indicating that the virus was incapable of causing illness after peripheral inoculation. Mice inoculated with YF/JE SA_{14}-14-2 developed neutralizing antibodies against JE virus (Fig. 7).

Construction of cDNA Templates for Generation of Yellow Fever/Dengue (YF/DEN) Chimeric Viruses

Derivation of chimeric Yellow Fever/Dengue (YF/DEN) viruses is described as follows, which, in principle, is carried out the same as construction of the YF/JE chimera described above. Other flavivirus chimeras can be

MON310 furnished by Dr. Wright. PCR primers included a 5' primer flanking the *Sph*I site and a 3' primer homologous to the DEN-2 nucleotides immediately upstream of the signalase site at the E/NSI junction and replacing the signalase site by substitutions that create a novel site, but also introduce a *Nar*I site. The resulting 1,170 basepair PCR fragment was then introduced into YFM5.2(*Nar*I[+]*Sph*I[-]).

The 5' portion of the DEN-2 clone including the prM and amino terminal portion of the E protein was engineered into the YF5'3'IV plasmid using a chimeric PCR primer. The chimeric primer, incorporating the 3' end of negative-sense YF C protein and 5' end of DEN-2 prM protein, was used with a positive-sense primer flanking the SP6 promoter of the YF5'3'IV plasmid to generate a 771 basepair PCR product with a 20 base extension representing DEN-2 prM sequence. This PCR product was then used to prime the DEN-2 plasmid in conjunction with a 3' primer representing DEN-2 sequence 1,501-1,522 and flanking the *Sph*I, to generate an 1,800 basepair final PCR product including the YF sequence from the *Not*I site through the SP6 promoter, YF 5' untranslated region, and YF C protein, contiguous with the DEN-2 prM-E1522 sequence. The PCR product was ligated into YF5'3'IV using *Not*I and *Sph*I sites to yield the YF5'3'IV/DEN(prM-E) plasmid.

Construction of Chimeric Templates for Other Flaviviruses

Procedures for generating full-length cDNA templates encoding chimeric YF/MVE, YF/SLE, YF/WN, YF/TBE viruses are similar to those described above for the YF/DEN-2 system. Table 6 illustrates the features of the strategy for generating YF17D-based chimeric viruses. The unique restriction sites used for *in vitro* ligation, and the chimeric primers for engineering the C/prM and E/NSI junctions are also shown. Sources of cDNA

this protein may abrogate the immunity to YF associated with antibodies against NS1, and thus avoid problems with vector immunity if more than one chimeric vaccine was to be needed in a given recipient, or if a YF vaccine had been previously given or needed at a future point.

The strategy involves creating a series of in-frame deletions within the NS1 coding region of the YFM5.2 plasmid, in conjunction with engineering a translational termination codon at the end of E, and a series of two IRESs (internal ribosome entry sites). One IRES is immediately downstream of the termination codon and allows for expression of an open reading frame within the region between E and NS1. The second IRES initiates translation from truncated NS1 proteins, providing expression of the remainder of the YF nonstructural polyprotein. These derivatives are tested for recovery of infectious virus and the construct with the largest deletion is used for insertion of foreign sequences (*e.g.*, HCV proteins) in the first IRES. This particular construct can also serve as a basis for determining whether deletion of NS1 will affect vector-specific immunity in the context of YF/Flavivirus chimeric viruses expressing prM-E, as described above.

The insertion of nucleotides encoding E1, E2, and/or E1 plus E2 HCV proteins is limited by the size of the deletion tolerated in the NS1 protein. Because of this, truncated HCV proteins can be used to enhance stability within the modified YF clone. The HCV proteins are engineered with an N-terminal signal sequence immediately following the IRES and a termination codon at the C terminus. This construction will direct the HCV proteins into the endoplasmic reticulum for secretion from the cell. The strategy for this construction is shown schematically in Fig. 6. Plasmids encoding HCV proteins of genotype I can be used for these constructions, for example, HCV plasmids obtained from Dr. Charles Rice at Washington University (Grakoui *et*

Table 1

Characterization of YF/JE chimeras

Clone	Yield (µg)	Infectivity plaques/100ng LLC-MK2	PBS log titer VERO	RNAse log titer VERO	DNAse log titer VERO
YF5.21v	5.5	15	7.2	0	7
YF/JE-S	7.6	50	6.2	0	6.2
YF/JE-N	7	60	5	0	5.4

Table 2

Sequence comparison of JE strains and YF/JE chimeras

Virus	E 107	E 138	E 176	E 177	E 227	E 243	E 244	E 279
JE SA14-14-2	F	K	V	T	S	K	G	M
YF/JE SA14-14-2	F	K	V	A	S	E	G	M
YF/JE NAK	L	E	I	T	P	E	E	K
JE NAK	L	E	I	T	P	E	E	K
JE SA14	L	E	I	T	S	E	G	K

Table 3

Plaque reduction neutralization titers on YF/JE chimeras

Virus	non-immune ascitic fluid	YF ascitic fluid	JE ascitic fluid	non-immune IgG	YF IgG
YF5.2iv	<1.3	3.7	<1.3	<2.2	>4.3
JE SA14-14-2	<1.3	<1.3	3.4	<2.2	<2.2
YF/JE SA14-14-2	<1.3	<1.3	3.1	<2.2	<1.9
YF/JE Nakayama	<1.3	<1.3	3.4	<2.2	<2.2

Table 6

Engineering of YF/Flavivirus chimeras

Virus	Chimeric C/prM junction[1]	Chimeric E/NS1 junction[2]	5' ligation[3]	3' ligation[4]	Sites[5] eliminated or (created)
YF/WN	X-cactgggagagcttgaaggtc (SEQ ID NO:1)	aaagccagttgcagccgcggtttaa (SEQ ID NO:2)	*Aat*II	*Nsi*I	
YF/DEN-1	X-aaggtagactggtgggctccc (SEQ ID NO:3)	gatcctcagtaccaaccgcggtttaa (SEQ ID NO:4)	*Aat*II	*Sph*I	*Sph*I in DEN
YF/DEN-2	X-aaggtagattggtgtgcattg (SEQ ID NO:5)	aaccctcagtaccaccgcggtttaa (SEQ ID NO:6)	*Aat*II	*Sph*I	
YF/DEN-3	X-aaggtgaattgaagtgctcta (SEQ ID NO:7)	acccccagcaccaccgcggtttaa (SEQ ID NO:8)	*Aat*II	*Sph*I	*Xho*I in DEN (*Sph*I in DEN)
YF/DEN-4	X-aaaaggaacagttgttctcta (SEQ ID NO:9)	acccgaagtgtcaaccgcggtttaa (SEQ ID NO:10)	*Aat*II	*Nsi*I	
YF/SLE	X-aacgtgaatagttggatagtc (SEQ ID NO:11)	accgttggtcgcaccgcggtttaa (SEQ ID NO:12)	*Aat*II	*Sph*I	*Aat*II in SLE
YF/MVE	X-aatttcgaaaggtggaaggtc (SEQ ID NO:13)	gaccggtgtttacagccgcggtttaa (SEQ ID NO:14)	*Aat*II	*Age*I	(*Age*I in YF)
YF/TBE	X-tactgcgaacgacgttgccac (SEQ ID NO:15)	actgggaacctcaccgcggtttaa (SEQ ID NO:16)	*Aat*II	AgeI	(*Age*I in YF)

1,2: The column illustrates the oligonucleotide used to generate chimeric YF/Flavivirus primers corresponding to the C/prM or E/NS1 junction. (See text). X = carboxyl terminal coding sequence of the YF capsid. The underlined region corresponds to the targeted heterologous sequence immediately upstream of the *Nar*I site (antisense - ccgcgg). This site allows insertion of PCR products into the Yfm5.2 (*Nar*I) plasmid required for generating full-length cDNA templates. Other nucleotides are specific to the heterologous virus. Oligonucleotide primers are listed 5' to 3'.

3,4: The unique restriction sites used for creating restriction fragments that can be isolated and ligated *in vitro* to produce full-length chimeric cDNA templates are listed. Because some sequences do not contain convenient sites, engineering of appropriate sites is required in some cases (footnote 5).

5: In parentheses are the restriction enzyme sites that must be created either in the YF backbone or the heterologous virus to allow efficient *in vitro* ligation. Sites not in parentheses must be eliminated. All such modifications are done by silent mutagenesis of the cDNA for the respective clone. Blank spaces indicate that no modification of the cDNA clones is required.

Other Embodiments

Other embodiments are within the following claims. For example, the prM-E protein genes of other flaviviruses of medical importance can be

response. The vaccine can be administered as a primary prophylactic agent in adults or children at risk of flavivirus infection. The vaccines can also be used as secondary agents for treating flavivirus-infected patients by stimulating an immune response against the flavivirus.

It may be desirable to use the yellow fever vaccine vector system for immunizing a host against one virus (for example, Japanese Encephalitis virus) and to later reimmunize the same individual against a second or third virus using a different chimeric construct. A significant advantage of the chimeric yellow fever system is that the vector will not elicit strong immunity to itself. Nor will prior immunity to yellow fever virus preclude the use of the chimeric vaccine as a vector for heterologous gene expression. These advantages are due to the removal of the portion of the yellow fever vaccine E gene that encodes neutralizing (protective) antigens to yellow fever, and replacement with another, heterologous gene that does not provide cross-protection against yellow fever. Although YF17D virus nonstructural proteins may play a role in protection, for example, by eliciting antibodies against NS1, which is involved in complement-dependent antibody mediated lysis of infected cells (Schlesinger *et al.*, J. Immunology 135:2805-2809, 1985), or by inducing cytotoxic T cell responses to NS3 or other proteins of the virus, it is unlikely that these responses will abrogate the ability of a live virus vaccine to stimulate neutralizing antibodies. This is supported by the facts that (1) individuals who have been previously infected with JE virus respond to vaccination with YF17D similarly to persons without previous JE infection, and (2) individuals who have previously received the YF17D vaccine respond to revaccination with a rise in neutralizing antibody titers (Sweet *et al.*, Am. J. Trop. Med. Hyg. 11:562-569, 1962). Thus, the chimeric vector can be used in populations that are immune to yellow fever because of prior natural infection or vaccination,

(Chambers *et al.* (1990) "Flavivirus Genome Organization, Expression, and Replication," In *Annual Review of Microbiology* 44:649-688), providing an important safety measure.

All references cited herein are incorporated by reference in their entirety.

What is claimed is:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACTGGGAGA GCTTGAAGGT C 21

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAAGCCAGTT GCAGCCGCGG TTTAA 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAGGTAGACT GGTGGGCTCC C 21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCCCCAGCA CCACCCGCGG TTTAA 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAAAGGAACA GTTGTTCTCT A 21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACCCGAAGTG TCAACCGCGG TTTAA 25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AACGTGAATA GTTGGATAGT C 21

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACTGGGAACC TCACCCGCGG TTTAA 25

mutation that prevents prM cleavage to produce M protein.

7. The chimeric virus of claim 1, wherein the signal sequences at the C/prM and E/NS1 junctions are maintained in construction of said chimeric flavivirus.

8. Use of a chimeric live, infectious, attenuated virus in the preparation of a medicament for preventing or treating flavivirus infection in a patient, wherein the chimeric, live, infectious attenuated virus comprises

a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or mutated so that functional yellow fever virus prM-E protein is not expressed, and

integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that said prM-E protein of said second flavivirus is expressed.

9. The use of claim 8, wherein said second flavivirus is a Japanese Encephalitis (JE) virus.

10. The use of claim 8, wherein said second flavivirus is a Dengue virus selected from the group consisting of Dengue types 1-4.

11. The use of claim 8, wherein said second flavivirus is selected from the group consisting of a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus, a Hepatitis C virus, a Kunjin virus, a Central European Encephalitis virus, a Russian Spring-Summer Encephalitis virus, a Powassan virus, a Kyasanur

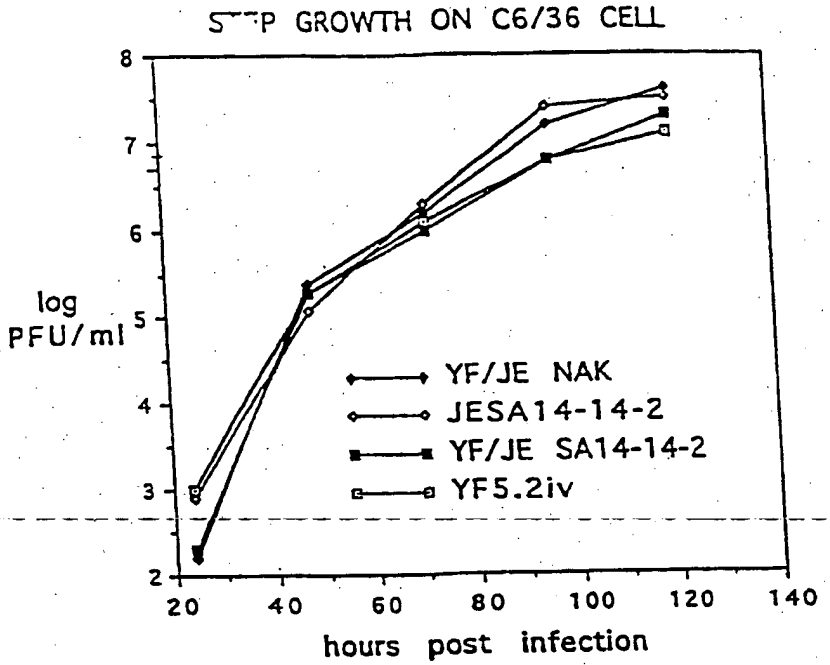
1-4.

18. The nucleic molecule of claim 15, wherein said second flavivirus is selected from the group consisting of a Murray Valley Encephalitis virus, a St. Louis Encephalitis virus, a West Nile virus, a Tick-borne Encephalitis virus, a Hepatitis C virus, a Kunjin virus, a Central European Encephalitis virus, a Russian Spring-Summer Encephalitis virus, a Powassan virus, a Kyasanur Forest Disease virus, and an Omsk Hemorrhagic Fever virus.

19. The nucleic acid molecule of claim 15, wherein the nucleotide sequence encoding the prM-E protein of said second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus.

20. The nucleic acid molecule of claim 15, wherein said nucleotide sequence encoding said prM-E protein of said second, different flavivirus comprises a mutation that prevents prM cleavage to produce M protein.

21. The nucleic acid molecule of claim 15, wherein the signal sequences at the C/prM and E/NS1 junctions are maintained in construction of said chimeric flavivirus.


S P GROWTH ON C6/36 CELL
log PFU/ml
8
7
6
5
4
3
2
20
40
60
80
100
120
140
hours post infection
YF/JE NAK
JESA14-14-2
YF/JE SA14-14-2
YF5.2iv

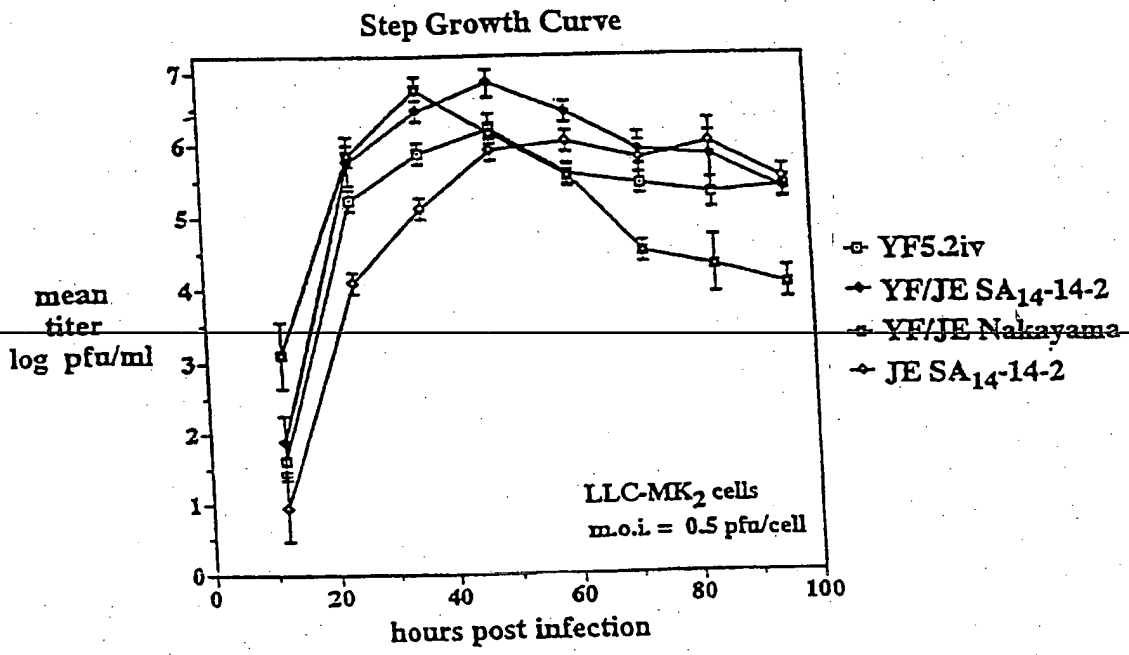
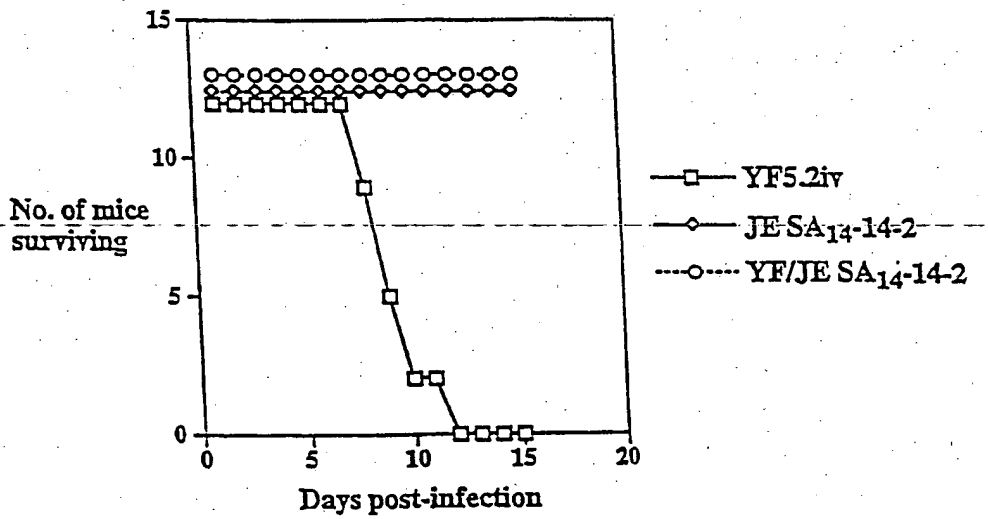

Step Growth Curve
mean titer log pfu/ml
7
6
5
4
3
2
1
0
0
20
40
60
80
100
hours post infection
LLC-MK2 cells
m.o.i. = 0.5 pfu/cell
YF5.2iv
YF/JE SA14-14-2
YF/JE Nakayama
JE SA14-14-2

FIG. 2

Mouse neurovirulence analysis

MICE: 4 week old ICR males/females
VIRUS DOSE: 10^4 pfu intracerebrally



Virus	Survival	P
YF5.2iv	0/12 (0%)	-
JE SA_{14}-14-2	12/12 (100%)	<0.001
YF/JE SA_{14}-14-2	13/13 (100%)	<0.001

Fig. 4

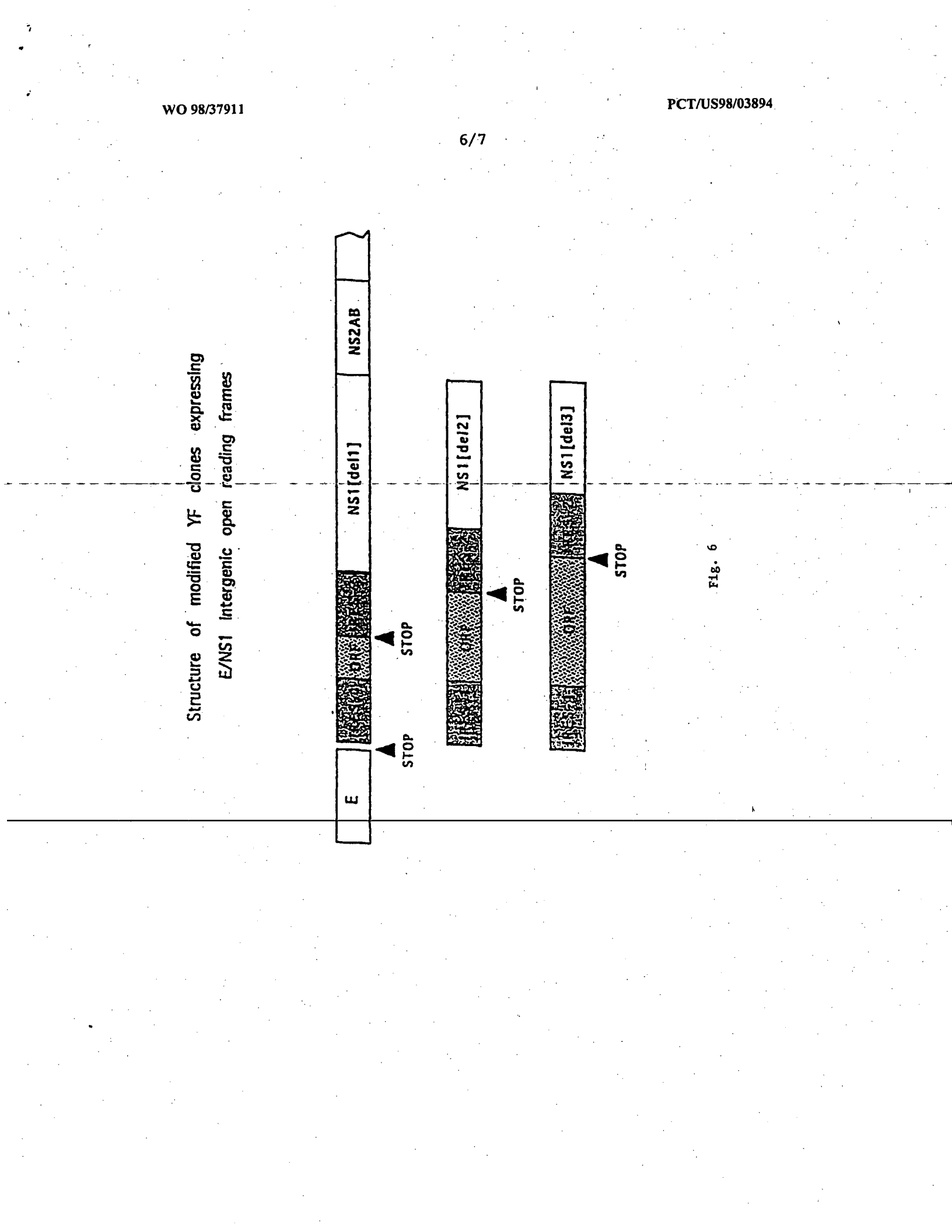

Structure of modified YF clones expressing
E/NS1 intergenic open reading frames
E
NS1[del1]
NS2AB
STOP
STOP
NS1[del2]
STOP
NS1[del3]
STOP

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03894

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/12, 39/193; C12N 7/01
US CL :424/199.1, 218.1; 435/235.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/199.1, 218.1; 435/235.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE
search terms:Japanese encephalitis, attenuat?, yellow fever, chimer?, dengue, flavivir?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BRAY et al. Construction of Intertypic Chimeric dengue Viruses by Substitution of Structural Protein Genes. November 1991. Proc. Natl. Acad. Sci., USA. Vol. 88, pages 10342-10346, see entire document.	1-21
Y	VENUGOPAL et al. Towards a New Generation of Flavivirus Vaccines. Vaccine. 1994. Vol. 12, No. 11, pages 966-975, see entire document.	1-21
Y	MARCHEVSKY et al. Phenotypic Analysis of Yellow Fever Virus Derived From Complementary DNA. American J Tropical Medicine & Hygiene. 1995. Vol. 52, No. 1, pages 75-80, see entire document..	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
24 APRIL 1998	23 JUN 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MP WOODWARD
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